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Research paper

The proteomic advantage: Label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis

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ABSTRACT

Coliform mastitis remains a primary focus of dairy cattle disease research due in part to the lack of efficacious treatment options for the deleterious side effects of exposure to LPS, including profound intra-mammary inflammation. To facilitate new veterinary drug approvals, reliable biomarkers are needed to evaluate the efficacy of adjunctive therapies for the treatment of inflammation associated with coliform mastitis. Most attempts to characterize the host response to LPS, however, have been accomplished using ELISAs. Because a relatively limited number of bovine-specific antibodies are commercially available, reliance on antibodies can be very limiting for biomarker discovery. Conversely, proteomic approaches boast the capability to analyze an unlimited number of protein targets in a single experiment, independent of antibody availability. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), a widely used proteomic strategy for the identification of proteins in complex mixtures, has gained popularity as a means to characterize proteins in various bovine milk fractions, both under normal physiological conditions as well as during clinical mastitis. The biological complexity of bovine milk has, however, precluded the complete annotation of the bovine milk proteome. Conventional approaches to reducing sample complexity, including fractionation and the removal of high abundance proteins, has improved proteome coverage, but the dynamic range of proteins present, and abundance of a relatively small number of proteins, continues to hinder comparative proteomic analyses of bovine milk. Nonetheless, advances in both liquid chromatography and mass spectrometry instrumentation, including nano-flow liquid chromatography (nano-LC), nano-spray ionization, and faster scanning speeds and ionization efficiency of mass spectrometers, have improved analyses of complex samples. In the current paper, we review the proteomic approaches used to conduct comparative analyses of milk from healthy cows and cows with clinical mastitis, as well as proteins related to the host response that have been identified in mastitic milk. Additionally, we present data that suggests the potential utility of LC–MS/MS label-free quantification as an alternative to costly labeling strategies for the relative quantification of individual proteins in complex mixtures. Temporal expression patterns generated using spectral counts, an LC–MS/MS label-free quantification strategy, corresponded well with ELISA data

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for acute phase proteins with commercially available antibodies. Combined, the capability to identify low abundance proteins, and the potential to generate temporal expression profiles, indicate the advantages of using proteomics as a screening tool in biomarker discovery analyses to assess biologically relevant proteins modulated during disease, including previously uncharacterized targets.

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1. Background

Mastitis caused by gram negative pathogens remains a principal focus of veterinary research due to staggering economic loss, the limited number of efficacious treatment options, and the lack of accurate biomarkers to evaluate the efficacy of new animal drugs proposed as adjunctive therapies. The need to better understand the host response to gram negative pathogens, and to identify reliable biomarkers specific to coliform mastitis, has led to several investigations into the soluble mediators of innate immunity in the bovine mammary gland (reviewed in Bannerman, 2009). Historically, characterization of the bovine innate immune response to LPS, and the quantification of changes in mediators of inflammation present in bovine milk during coliform mastitis has, however, been dominated by the use of ELISAs. While ELISAs feature both accuracy and specificity, antibody-based strategies are restricted by the ability to detect and quantify only one protein at a time, and by a reliance on the availability or development of species-specific antibodies. ELISAs, therefore, have little application to the discovery of novel inflammatory mediators, as currently only a limited number of bovine-specific antibodies are commercially available.

Proteomics, defined as the identification and characterization of all proteins within a cell or tissue (Colantonio and Chan, 2005), boasts a significant advantage over ELISAs in that proteomics involves the use of analytical methodologies, such as liquid chromatography (LC) and mass spectrometry (MS), to isolate, identify, and characterize proteins, and is not reliant on the use or availability of antibodies. The use of proteomics is also much less restrictive than ELISAs in that theoretically, an unlimited number of proteins can be analyzed simultaneously in a given experiment using proteomic strategies. Furthermore, advances in soft ionization techniques in mass spectrometry, including electro-spray ionization (ESI), nano-spray ionization, and matrix-assisted laser desorption/ionization (MALDI), have broadened the applications of mass spectrometry to include the characterization of biopolymers such as intact proteins and peptides (reviewed in Mann et al., 2001).

Previous studies have utilized proteomic strategies in attempts to identify protein biomarkers of the host response present in whey from bovine milk during mastitis (Boehmer et al., 2008, 2010; Smolenski et al., 2007; Hogarth et al., 2004). In most cases, however, a limited number of low abundance proteins have been robustly identified in comparative proteomic analyses of the bovine milk proteome. Better characterization of extremely low abundance proteins in bovine milk following infection with a gram negative pathogen was of specific interest to our group, because the assessment of the modulation in low abundance proteins during disease could prove useful in the establishment of biomarkers of coliform mastitis for use both as diagnostic tools, and as indicators of drug efficacy.

Biomarker discovery in bovine milk has, however, been hindered both by prominent proteomic bottlenecks, as well as other experimental factors. The most significant factor that has precluded the identification of a larger number of low abundance proteins related to host response in milk, and one of the most common obstacles in proteomic analyses, is the biological complexity of the matrix. The analytical challenges associated with the complexity of milk include protein proteolysis, the numerous post-translational modifications (PTMs) that occur in milk proteins including glycosylation, phosphorylation, and disulfide bond formation, as well as the dynamic range of proteins in milk (Gagnaire et al., 2009; O'Donnell et al., 2004). The profound relative abundance of a limited number of proteins in bovine milk is arguably the most challenging aspect of the proteomic analysis of milk, as the presence of abundant proteins often prohibits the robust identification of low abundance components. Comparative analyses of bovine milk are further confounded by the dynamic range of proteins present in milk, because milk collected from healthy cows is characterized by the abundance of the caseins and whey proteins β-lactoglobulin and α-lactalbumin, while milk collected from cows with coliform mastitis is marked by the profound increase in vascular-derived proteins, most notably serum albumin (Fig. 1).

Conventional approaches to reducing sample complexity prior to analysis, including the selective depletion of high abundance proteins and fractionation of samples, have not yet been effectively adapted to address the specific complexities of bovine milk. For example, attempts to remove high abundance proteins, including casein depletion by acid precipitation (Hogarth et al., 2004) and the removal of immunoglobulins by immunoaffinity (Yamada et al., 2002), have resulted in a rather drastic reduction in the number of milk proteins identified when compared to proteomic analyses of bovine milk that did not involve removal of high abundance proteins (Boehmer et al., 2008; Smolenski et al., 2007). An additional complication related to the comparative analyses of bovine milk is that multiple strategies are required to reduce the complexity of normal versus mastitic milk samples, as the dynamics of protein abundance change during inflammation. Likewise, it has been demonstrated that the removal of serum albumin, which would be necessary to reduce the complexity of mastitic milk samples, can lead to the non-specific depletion of low abundance proteins (Gundry et al., 2007), which could interfere with biomarker discovery.

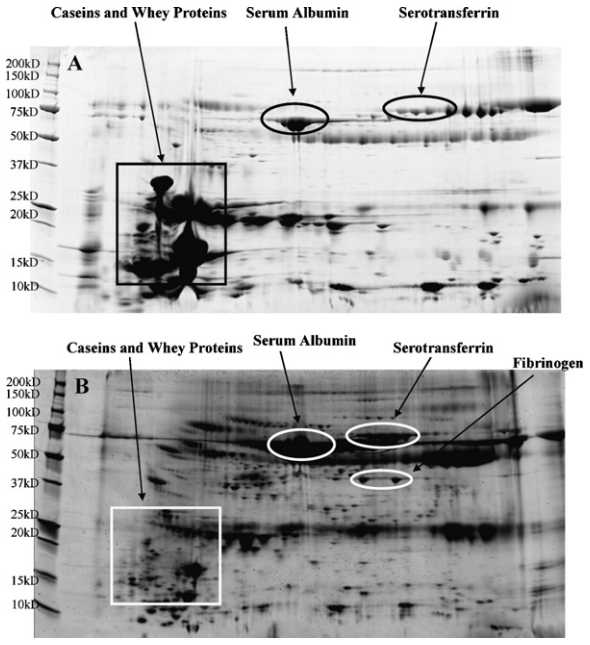

Fig. 1. Differential protein expression in whey from normal milk (A) and whey from mastitic milk (B) profiled using 2-dimensional gel electrophoresis (Boehmer et al., 2008). Whey from normal milk is characterized by the abundance of the casein and whey proteins, while whey from mastitic milk, in contrast, is characterized by the increased abundance of serum albumin and other vascular-derived proteins including serotransferrin and fibrinogen.

Independent of matrix complexity, another critical aspect when considering biomarker discovery and the utilization of biomarkers to evaluate disease progression, or the efficacy of a drug treatment, is the evaluation of protein changes during the course of clinical disease (Simpson et al., 2009; Mueller et al., 2008; Old et al., 2005). Regrettably, most prior studies of protein modulation in the bovine milk proteome during disease focused solely on normal versus mastitic milk, rather than changes in protein abundance over the course of infection (Boehmer et al., 2008; Smolenski et al., 2007; Hogarth et al., 2004).

In biomarker discovery, however, there is still no "gold standard" for the accurate quantification of individual proteins in complex biological samples using proteomic strategies, especially for proteins present in low abundance (Mueller et al., 2008). Several labeling strategies are available for the quantification of proteins in conjunction with LC–MS/MS analyses, but labeling strategies can be cost limiting, often require pair wise comparisons which can be problematic when quantifying proteins that are only present in a given physiological state, and labeling strategies do not allow for retrospective quantification (Old et al., 2005). Consequently, label-free methods for the relative quantification of proteins in complex biological samples have been investigated, including the use of ion intensity, the number of unique peptides assigned to a given protein, and spectral counts as measures of relative abundance for individual proteins in a complex sample (Old et al., 2005; Zybailov et al., 2005; Liu et al., 2004). Label-free methods have gained popularity primarily because there are no associated costs, normalization can allow for comparisons of protein abundance across a longitudinal set of samples, and analyses can be conducted retrospectively (Mueller et al., 2008). A recent study conducted by our group focused on changes in the relative abundance of milk proteins over the course of an experimentally induced coliform infection using peptide counts, a label-free strategy, but the proteins evaluated were mainly medium to high abundance proteins (Boehmer et al., 2010).

In addition to expanding our knowledge of the bovine milk proteome, an added objective was to further evaluate the feasibility of using label-free LC–MS/MS strategies as a screening tool to identify biologically relevant proteins modulated during disease, especially low-abundance proteins, and proteins for which there are no available antibodies. To assess the validity of using spectral counts to quantify changes in proteins for which no antibody has been developed, we conducted a comparison of the expression of milk proteins determined using LC–MS/MS data with expression profiles generated using an ELISA, similar to comparisons made in our previous studies (Boehmer et al., 2010).

A second interest was the evaluation of samples collected over the course of infection from several biological replicates. Our aim was the discovery of a reproducible biomarker or pattern of biomarkers that presented in several biological replicates, as consistent patterns could suggest both a response that was indicative of coliform mastitis, as well as the time frame following infection during which the potential biomarker or biomarkers could be accurately monitored. Accordingly, we sought to determine if refinements in proteomic methodology, including investigations into utilizing more advanced approaches such as a mass spectrometer with a faster scanning speed and the ability to trap ions, and the use of nano-flow liquid chromatography in-line with nano-spray ionization, could enable the identification and characterization of a greater number of low abundance proteins when compared to earlier analyses of whey from mastitic bovine milk (Boehmer et al., 2008, 2010; Smolenski et al., 2007; Hogarth et al., 2004). Following recent analyses detailed in the current paper, the number of low abundance proteins identified suggests that proteomics could lead to a more thorough annotation of the bovine milk proteome. Additionally, the close correspondence of LC–MS/MS label-free data and ELISA data was a positive indication that proteomic strategies could serve as valuable screening tools for biomarker discovery, as well as the establishment of biomarkers specific to coliform mastitis.

2. Proteomic tools, strategies and challenges

Protein identification through the use of mass spectrometry can be divided into two main categories, referred to as top-down and bottom-up. The primary distinguishing features between the two main proteomic approaches is the isolation and fragmentation of intact proteins using mass spectrometry in a top-down approach, versus proteolytic digestion of mixtures of proteins, and the subsequent separation and fragmentation of peptides, in bottom-up proteomics. Identification of proteins in complex biological

mixtures using bottom-up proteomics is reliant upon the measurement of the masses of the peptides that are generated following proteolytic cleavage of the proteins. The mass of a peptide is determined using mass spectrometry, and is based upon a mass: charge ratio (m/z). Charged peptides are generated as a result of ionization, or the addition of a proton to the peptide, which results in the conversion of the peptide into an ion. The two most popular forms of ionization used in bottom-up proteomic analyses are ESI and MALDI. The primary features that distinguish MALDI from ESI are the matrices used for ionization, the charge states of the ions generated, and the actual mechanisms of ion formation characteristic of each method (reviewed in Mann et al., 2001). Similar in concept and ion formation to ESI, nanospray is another ionization method that has become very popular in proteomic analyses in recent years. The primary distinctions between ESI and nanospray are the significantly lower flow rates and smaller needle diameters used for nano-spray. An advantage of nano-spray ionization is that nano-spray sources can accommodate much lower flow rates than ESI, down to fractions of microliters per minute (Wilm and Mann, 1996). Additionally, droplet formation occurs more readily using nano-spray, resulting in increased ionization efficiency. The lower flow rates in the nanospray technique also allow for a longer length of analysis time, which leads to fewer missed peptides eluting off the chromatographic column while the mass spectrometer is engaged in MS/MS scans (Wilm and Mann, 1996).

Since the invention of soft ionization techniques, bottom-up proteomic strategies including the use of LC to separate peptides coupled with MS/MS for peptide sequencing, a process commonly referred to as LC–MS/MS, has become the most extensively applied bottom-up proteomic approach for the identification of individual proteins in complex mixtures. LC–MS/MS involves proteolytic digestion of complex protein mixtures followed by the separation of peptides using one- or two-dimensional LC, and analysis of the peptides by MS/MS (reviewed in Mann et al., 2001). An enzyme commonly used to cut proteins into peptides is tryspin, which digests at the amino acid residues arginine and lysine. Peptide mixtures are separated, prior to introduction into the mass spectrometer for mass analysis, by passage over a chromatographic column and subsequent separation based on either charge, called ion exchange LC, or hydrophobicity, which is termed reverse phase (RP) LC. The number of proteins identified using LC–MS/MS is directly dependent on the efficiency of peptide separation (reviewed in Mann et al., 2001). In data-dependent acquisitions, the mass spectrometer is programmed to scan the masses of ionized peptides and to select anywhere from 3 to 10 most abundant peptides for further fragmentation by collision-induced dissociation (CID). An inert gas introduced into the collision cell of the mass spectrometer during CID induces fragmentation of the peptides, a process which results in the production of a tandem mass spectrum. Peak lists distilled from tandem mass spectra are used to query against an MS/MS spectra database to determine peptide identity, and the sequenced peptides are assigned to proteins for protein identification.

When the chromatographic separation of peptides is poor, the potential for selection of a peptide from a low abundance protein for CID will decrease, due to the fact that peptides from dominant proteins will be in greater numbers in the sample and will be preferentially selected for further fragmentation. Additionally, poorly resolved peptides tend to co-elute off the chromatographic column into the mass spectrometer, a phenomena which leads to CID of more than one peptide at a time. The tandem mass spectrum that results from co-eluting peptides thus represents the fragmentation of a peptide mixture, and will often fail to yield a match when searched against a protein database, or will lead to a false positive peptide assignment.

2.1. Biological complexity and proteomic bottlenecks

Proteomic strategies, though capable of analyzing a theoretically unlimited number of proteins in a single experiment, are not devoid of challenges. Post-translational modifications (PTMs) of proteins in a given proteome, and the dynamic range of proteins present in the sample, are direct reflections of the complexity of the biological matrix, and can pose significant roadblocks to protein identification. Dynamic range is one of the most prominent bottlenecks in proteomic experiments because many biological samples, including milk, are characterized by the presence of a select number of highly abundant proteins that account for a large percentage of the total protein concentration in the sample, and numerous low abundance proteins that comprise a very small percentage of protein concentration (Gagnaire et al., 2009; O'Donnell et al., 2004). Given the fact that abundant proteins are often affiliated with a variety of biological functions and pathways, and thus rarely meet the specificity criteria necessary to be termed a biomarker of disease, the removal or depletion of abundant proteins is a common first step in proteomic analyses aimed at biomarker discovery.

In some cases, however, removal of abundant proteins from a complex matrix can also result in the non-selective depletion of low abundance proteins, a consequence which can cause the loss of potentially relevant diagnostic, clinical, and biological information. Albumin, which accounts for nearly 55% of the total protein concentration of plasma, is often targeted for removal prior to proteomic analysis. Investigation into the albuminome, or the number of proteins that bind to, or are associated with, albumin in plasma and are thus depleted along with the abundant protein following the application of depletion strategies, revealed that as many as 35 high and low abundance proteins were bound to and removed along with albumin following an affinity removal process (Gundry et al., 2007). The increased concentration of albumin in milk during coliform mastitis, due to the breakdown of the blood–milk barrier following exposure to LPS, presents a significant analytical roadblock for the identification of low abundance proteins. Abundant albumin peptides often mask the detection of low abundance proteins, and albumin depletion could result in the loss of low abundance proteins that potentially bind to albumin in milk.

There are many strategies available commercially that are designed to remove or deplete high abundance proteins, most notably serum albumin, from biological samples in order to enhance the detection of low abun-

dance proteins. Albumin removal strategies often involve an analytical column or disc pre-packed with a form of Cibacron Blue, a sulfonated triazine dye used for affinity chromatography, immobilized onto a support matrix (Angal and Dean, 1977). For the removal of several high abundance proteins simultaneously, multiple affinity removal system (MARS) affinity spin and chromatographic columns are commercially available from Agilent Technologies. Low abundance protein enrichment strategies are also commercially available, including a widely used product from Bio-Rad Laboratories called ProteoMiner. ProteoMiner columns consist of beads containing a highly diverse library of hexapeptides, each with a specific protein affinity, bound to chromatographic supports. The theory behind ProteoMiner is that proteins in a given biological sample, when passed over the beads, will bind to specific ligands, out-competing high abundance proteins, and allowing excess proteins to wash off the column as flow-through. Proteins bound to the beads on the ProteoMiner sample column are eluted, and the resulting protein pool is predicted to contain a more equivalent representation of both high and low abundance proteins present in the given sample. Nearly all of the depletion, removal, or enrichment strategies that are available, however, have been optimized, and are intended, for use with human serum or plasma. Used according to the manufacturer's recommended protocol, the ProteoMiner enrichment strategy was extremely effective at depleting serum albumin from mastitic milk samples, but also resulted in the depletion of several other milk proteins (Fig. 2). While the enrichment for low abundance targets in mastitic milk was effective for proteins between 10 and 15 kD, most of the spots in that range were identified as proteolysis products of the casein proteins (data not shown). In order to make effective use of commercially available strategies for the reduction of sample complexity, optimization would have to be performed for milk samples specifically, which might not be economically feasible for some studies, given the high cost of many of the kits.

In addition to serum albumin, several proteins whose presence in blood increases during the inflammatory response such as complement, clotting factors, adhesion molecules, and acute phase proteins increase in concentration in milk during coliform mastitis, due to the well characterized break-down of the blood–milk barrier. Many of the proteins that leak into the milk from systemic circulation are very large glycoproteins that become heavily modified during the course of infection (Kjeldsen et al., 2003; Soerensen et al., 1995), an aspect that further complicates analytical challenges. Accurate identification of modified proteins can require specialized sample preparation prior to analysis, the inclusion of numerous potential variable modifications when querying peak lists against protein databases, or the use of fragmentation strategies other than CID, including electron-transfer dissociation (ETD).

While sample complexity reduction strategies may not be feasible approaches for enriching low abundance targets in milk, proteomic capabilities, including both instrumentation and fractionation options, continue to advance. Utilizing the features of different instrument systems, such as mass spectrometers with faster scanning speeds and increased ion transmission capabilities, in lieu of instruments with higher mass accuracy, could lead to more protein identifications, as well as targets for more focused analyses aimed at quantification or mass accuracy. Nano-flow liquid chromatography in-line with a mass spectrometer equipped with a nano-spray ionization source could likewise result in more robust identification of low abundance proteins, as nano-spray ionization has demonstrated advantages over traditional ESI for protein identification (Juraschek et al., 1998). Advances in LC and nano-flow LC, including two-dimensional LC separation strategies, could also drastically improve peptide separations and lead to additional protein identifications.

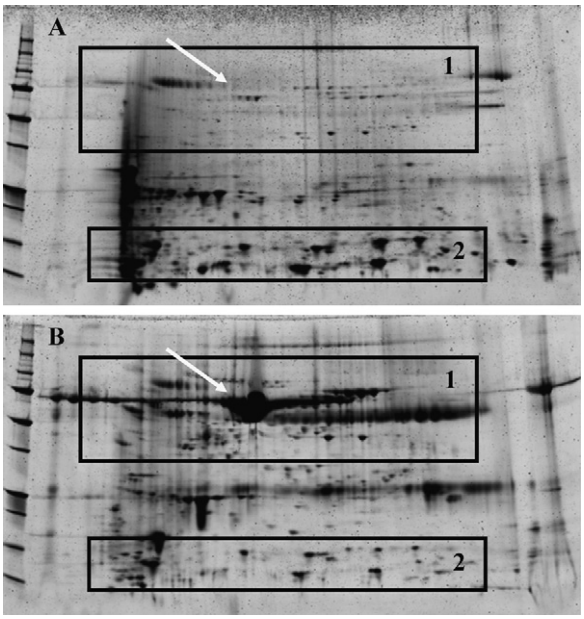

Fig. 2. The two dimensional profiles of whey from mastitic bovine milk following protein enrichment using ProteoMiner (BioRad Laboratories). The abundance of the protein serum albumin, indicated in box 1 with the white arrow, is clearly lower in the profile of proteins eluted off the beads containing a highly diverse library of hexapeptides (A) when compared to the profile of the proteins in the flow-through that did not bind to the beads (B). Likewise, smaller proteins at the bottom of the gel, in box 2, appeared to be more abundant in the protein pool eluted off the beads (A) when compared to the column flow through (B). Also apparent is the fact that the majority of the proteins appear in the flow through (B), as opposed to being enriched by binding to the beads (A). The use of strategies such as ProteoMiner, which was developed and optimized for use with plasma and serum samples, may not be feasible or may require added optimization steps, when used on complex biological samples such as bovine milk.

2.2. Quantification

An important criterion for the establishment of quality biomarkers is reliable quantification. Accordingly, relative and absolute quantification of changes in biomarkers in biological matrices using proteomic strategies is a topic that has garnered significant attention in recent years (Simpson et al., 2009; Mueller et al., 2008; Fenselau, 2007; Roe and Griffin, 2006). Quantification methods can

be assigned to one of two broad categories: a labeling approach that requires the incorporation of labels into proteins or peptides prior to MS analysis, or the use of a label-free method (Simpson et al., 2009).

The basis of most labeled quantification methods is the theory that a labeled peptide will behave chemically in the same fashion as its unlabeled counterpart, and will have identical chromatographic and MS properties (Simpson et al., 2009). The addition of a label, however, does result in a mass difference between the two peptides, and thus relative abundance can be inferred by comparing the respective signal intensities of the labeled and unlabeled peptides in the same MS run (Simpson et al., 2009). Labels can be incorporated in several ways, with the most popular being either metabolically, chemically, or enzymatically (Simpson et al., 2009; Fenselau, 2007). A popular means of metabolically incorporating a label is stable isotope labeling by amino acids in cell culture, or SILAC. The primary advantage of SILAC is that isotope labels can be incorporated into all cellular proteins, because cells are cultured with nutrients that are highly enriched with stable isotopes (Jiang and English, 2002; Gu et al., 2002; Oda et al., 1999). Different cell populations can then be combined following labeling in culture, and the relative abundances of individual proteins in the distinct cell populations determined by measuring the isotope ratios of peptide pairs with mass spectrometry (Fenselau, 2007). Metabolic labeling is, however, only applicable to the analysis of bacterial and eukaryotic cells, and cannot be adapted to accommodate clinical or animal studies.

Other labeling strategies that can be extended to a wider variety of biological samples include proteolytic labeling with O^{18}, in which two atoms of O^{18} are added to the carboxylic acid group of every peptide in a protein pool following proteolysis (Yao et al., 2003). Similar to SILAC, relative protein concentrations are determined by measuring isotope ratios of O^{18} and O^{16} labeled peptide pairs with mass spectrometry. Stable isotopes can also be incorporated by chemical derivatization at either the protein or peptide level. Two popular examples of labeling strategies that are based on chemical reactions are isotope coded affinity tags (ICAT; Gygi et al., 1999), and isobaric tags for relative and absolute quantification (iTRAQ; Ross et al., 2004). Both ICAT and iTRAQ are based on the derivatization of the primary amine groups of proteins or peptides. Using ICAT, samples are either labeled with an isotopically light probe or an isotopically heavy version, combined, digested with a protease (trypsin), and then labeled peptides are isolated by avidin affinity chromatography and analyzed by LC–MS (Gygi et al., 1999). Using iTRAQ, however, enables users to comparatively analyze four or more different protein pools simultaneously, unlike most labeling strategies that are limited to the analysis of two distinct protein populations (Ross et al., 2004). With iTRAQ, the proteins or peptides from different samples or treatments are derivatized with different tags that all have the same total mass. The labeled peptides are then pooled and analyzed using LC–MS/MS. Peptides from the different protein pools can be differentiated and relatively quantified following the fragmentation of the attached tag which generates a low molecular mass reporter ion (Ross et al., 2004). Because of the number of differential protein pools that can be simultaneously analyzed, iTRAQ is a very popular labeling strategy commonly used in proteomic screens, including the analysis of mastitis pathogens (Lippolis et al., 2009), the bovine milk fat globular membrane (Reinhardt and Lippolis, 2008), and bovine milk following *in vivo* LPS challenge (Danielsen et al., 2010).

In comparative proteomic analysis of normal versus mastitic milk aimed at biomarker discovery, however, labeling strategies are not always feasible for protein quantification, depending on the design and focus of the study. Labeling strategies can be extremely cost-limiting, which leads to the analysis of only a small number of samples from a limited number of biological replicates (Danielsen et al., 2010), and the buffers that are required during the labeling steps are not always amenable to maintaining all of the proteins present in milk in solution. Additionally, the majority of labeling strategies are based on the pair-wise comparison of the relative intensities of peptides generated from a target protein in two different physiological states (i.e. healthy versus diseased). Since many of the target proteins in comparative proteomic analyses of mastitic milk are not present in normal or control samples of milk, but only during a mastitis infection, accurately comparing the abundance of a peptide that is present at one physiological state but not the other is problematic. Because labeling strategies are well adapted to targeted analyses but not discovery screens, an interest in quantification without the incorporation of labels has emerged in the field of proteomics.

Label-free strategies are based on the correlation between the abundance of a protein or peptide in a sample, and the MS signal (Simpson et al., 2009). One of the most popular methods of label-free quantification is ion intensity, determined using extracted ion chromatograms (XIC), in which the number and intensity of selected precursor ions at a particular m/z are summed, and the peak areas used as a measure of relative abundance (Old et al., 2005). An alternative approach that is gaining popularity, however, is spectral counting, or the number of MS/MS spectra that contribute to the identification of a given protein (Zybailov et al., 2005; Liu et al., 2004). The theory behind spectral counting in particular is that the abundant proteins, when proteolytically digested, will yield numerous copies of the same peptide. Furthermore, the probability that abundant peptides will trigger multiple MS/MS events is higher than the likelihood of repeatedly sampling a peptide from a lower abundance protein. In previous investigations into the accuracy and linearity of spectral counts, the spectral counts for peptides from proteins spiked into yeast samples at known concentrations exhibited linearity over 2 orders of magnitude, and were highly correlated to relative protein abundance (Liu et al., 2004). The number of unique peptides identified for each protein in a sample has likewise been used as a measure of relative protein abundance (Liu et al., 2004). Similar to the theory behind the utility of spectral counts as a measure of relative protein abundance, the assumption with number of unique peptide assignments is that more abundant proteins will naturally have greater sequence coverage than lower abundance proteins, based on the fact that a greater

number of peptides from abundant proteins will be available for MS/MS sampling in a given peptide pool.

The inherent drawbacks of label-free methods, however, include the assumption that the linearity of response will be the same for each protein, which often does not hold true because the chromatographic behavior of peptides tend to vary (Simpson et al., 2009). Likewise, the amino acid composition of every peptide differs, and as a result, the ionization potential of each peptide generated in a bottom-up proteomic experiment is unique. Thus, some peptides will naturally ionize more efficiently than others, regardless of abundance. Additionally, label-free strategies are almost exclusively based on the identification of peptides that are unique to a given protein. In the instance of highly conserved proteins that share a specific functional domain, such as the iron binding domain present in both lactoferrin and transferrin, differentiation of peptides belonging to closely related proteins can be challenging unless the mass spectrometer employed in the analyses offers increased sensitivity and high mass accuracy. Normalization of XIC, and spectral or peptide counts, however, can help account for differences in chromatography and ionization potentials of peptides when using label-free methods (Old et al., 2005). Finally, label-free quantification does not require any extra sample processing and can be performed retrospectively; two attributes which make non-labeled quantification methods the continued focus of development in biomarker discovery research (Simpson et al., 2009).

3. Comparative proteomic analysis of whey from bovine milk

3.1. Two-dimensional gels with MALDI-TOF mass spectrometry

Some of the earliest comparative proteomic analyses of normal versus mastitic whey from bovine milk were accomplished using 2-dimensional gel electrophoresis (2D-GE) followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Smolenski et al., 2007; Hogarth et al., 2004), or MALDI-TOF/TOF post-source decay (PSD; Boehmer et al., 2008). The number of proteins identified in the studies that utilized 2D-GE followed by MALDI-TOF mass spectrometry (MS) was thirty-one, sixteen of which were detected only in whey from milk collected from cows with clinical mastitis (Table 1). Though somewhat limited, results of the 2D gel-based comparative proteomic analyses of normal versus mastitic whey from milk did contribute to our overall knowledge of protein modulation during mastitis, and extended the number of proteins related to host response identified in milk during a mastitis infection. Interestingly, the two studies that did not attempt the removal of high abundance proteins prior to analysis (Boehmer et al., 2008; Smolenski et al., 2007) resulted in the identification of a greater overall number of proteins, including a higher number of proteins related to the host response, than the study that attempted the

Table 1
Summary of proteins detected in bovine milk using MALDI-TOF MS.

Swiss-Prot entry name	Primary accession number	Protein name	Condition present[a]	Reference[b]
PIGR_BOVIN	P81265	Polymeric-immunoglobin receptor	N, M	1, 3
ALBU_BOVIN	P02769	Serum albumin	N, M	1, 2, 3
CASA1_BOVIN	P02662	α-S1-casein	N, M	1, 2, 3
CO3_BOVIN	Q2UVX4	Complement C3	N, M	1
MFGM_BOVIN	Q95114	Lactadherin	N	1, 3
CASK_BOVIN	P02668	κ-Casein	N	1, 2, 3
CASB_BOVIN	P02666	β-Casein	N	1, 2, 3
LACB_BOVIN	P02754	β-Lactoglobulin	N	1, 2, 3
NPC2_BOVIN	P79345	Epididymal secretory protein E1 precursor	N	1
LALBA_BOVIN	P00711	α-Lactalbumin	N	1, 2, 3
TTHY_BOVIN	O46375	Transthyretin	N, M	1
B2MG_BOVIN	P01888	β-2-Microglobulin	N	1
FABPH_BOVIN	P10790	Fatty acid-binding protein, heart	N	1
A1AG_BOVIN	Q3SZR3	α-1-Acid glycoprotein	N, M	1
TRFE_BOVIN	Q29443	Serotransferrin	M	1, 2, 3
TRFL_BOVIN	P24627	Lactoferrin	M	3
A1AT_BOVIN	P34955	α-1-Antitrypsin	M	1
FETUA_BOVIN	P12763	α-2-HS-glycoprotein	M	1
FIBB_BOVIN	P02676	Fibrinogen β-chain	M	1
CO4_BOVIN	P01030	Complement C4 precursor	M	1
APOA1_BOVIN	P15497	Apolipoprotein A-I precursor	M	1
CTHL1_BOVIN	P22226	Cyclic dodecapeptide precursor	M	1
GLCM1_BOVIN	P80195	Glyearn-1	N, M	3
CTHL4_BOVIN	P33046	Indolicidin precursor	M	1
CTHL3_BOVIN	P19661	Bactenecin-7 precursor	M	1
CTHL2_BOVIN	P19660	Bactenecin-5 precursor	M	1
APOA2_BOVIN	P81644	Apolipoprotein A-II precursor	M	1
ACTB_BOVIN	P60712	Actin, cytoplasmic 1	M	3
NUCB1_BOVIN	Q0P569	Nucleobindin	M	3
APOC3_BOVIN	P19035	Apolipoprotein C-III	M	1
S10AC_BOVIN	P79105	Protein S100-A12	M	1

[a] Condition present; N = normal bovine milk; M = mastitic bovine milk.
[b] References; 1 = Boehmer et al. (2008); 2 = Hogarth et al. (2004); 3 = Smolenski et al. (2007).

depletion of the highly abundant caseins (Hogarth et al., 2004).

Other promising discoveries that resulted from the 2D-GE of whey from bovine milk included the identification of the acute phase protein (APP) α-1-acid glycoprotein (A1AG) in both normal and mastitic whey samples, and the apparent higher relative abundance of A1AG in mastitic milk (Boehmer et al., 2008). Previous analyses of APP expression in milk during bovine mastitis had only identified the acute phase proteins SAA, lipopolysaccharide binding protein (LBP), and haptoglobin (Hiss et al., 2004; Bannerman et al., 2004; Eckersall et al., 2001). In addition to the identification of A1AG, proteins with antimicrobial properties not identified in other comparative proteomic analyses of bovine milk were discovered, including several members of the cathelicidin family of cationic antimicrobial proteins (Boehmer et al., 2008). However, despite the identification of proteins related to host response, including previously unreported acute phase and antimicrobial proteins, a very limited number of proteins were identified using 2D-GE coupled with MALDI-TOF MS. Additionally, though 2D-GE is a popular method for separation and quantifying proteins in complex biological samples, the electrophoresis approach and quantification of proteins via densitometry alone is limited in sensitivity, and problematic for proteins that are insoluble or have either a very high or low molecular weight (Old et al., 2005). Likewise, protein recovery from in-gel tryptic digestion can be limited, which reduces the probability of robust protein identification using MALDI-TOF MS. Lastly, the poor reproducibility of 2D gels, and the time required to generate 2-dimensional protein profiles do not lend well to high throughput biomarker discovery or to the analysis of protein modulation over the course of clinical mastitis.

3.2. LC–MS/MS of whey from normal and mastitic bovine milk

In total, 71 proteins (Table 2) have been identified in comparative analyses of whey from bovine milk using bottom-up LC–MS/MS strategies (Danielsen et al., 2010; Boehmer et al., 2008, 2010; Smolenski et al., 2007). The very first LC–MS/MS-based proteomic analysis of whey, skim milk, and the milk fat globular membrane from mastitic bovine milk resulted in the identification of 24 defense-related proteins, in addition to the abundant casein and whey proteins (Smolenski et al., 2007). Of the 24 proteins identified, however, only 6 proteins related to the host response were identified in whey using direct LC–MS/MS. Additionally, a major limitation of the study was the fact that milk from only one cow with a naturally occurring case of clinical mastitis was analyzed, and the study was not comparative, as proteins were only identified in mastitic milk and not in normal milk from the same cow. Despite the lack of biological replicates and control milk samples, however, the study marked the first comparative analyses of several different mastitic milk fractions, and was the first to report the identification of several low abundance milk proteins (Smolenski et al., 2007).

In our first proteomic analyses of a longitudinal series of bovine milk samples collected from 8 mid-lactation cows before and over the course of experimental infection with *E. coli*, a traditional bottom-up LC–MS/MS approach was applied using an ultra pressure LC instrument (UPLC) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Boehmer et al., 2010). Similar to earlier analyses, only 6 proteins related to host response were identified in whey from milk following *E. coli* challenge, including transferrin, lactoferrin, and the cationic antimicrobial proteins cathelicidins-1 and peptidoglycan recognition receptor protein. Nonetheless, temporal expression of the proteins identified in whey from milk before, and in the time points following the experimental induction of coliform mastitis, was evaluated using both the number of unique peptides assigned to each identified protein, and the spectral count, or the number of times each peptide triggered an MS/MS event. The number of unique peptides identified and the spectral counts were nearly identical for each protein identified which, in combination with the extremely limited number of proteins identified, indicated that the analytical methods lacked adequate sensitivity. Additionally, there was a large gap in the number of peptides identified for abundant whey proteins, and the number of peptide assignments for the remaining proteins identified (Boehmer et al., 2010).

Due to the inherent lack of sensitivity achieved using the previously described instrument system (Boehmer et al., 2010), whey from bovine milk before and after challenge with *E. coli* was analyzed using one-dimensional nano-LC followed by nano-spray tandem MS. A linear ion-trap mass spectrometer was chosen in order to evaluate the advantage of the fast scanning speed of the instrument, and nano-spray was applied to potentially improve ionization efficiency. Across 8 biological replicates, a total of 33 different proteins were detected in milk samples collected from 4 or more biological replicates and at a minimum of 2 of the 5 time points analyzed (Table 3). Of the 33 proteins identified, 12 proteins were detected across all time points, while 21 were detected only following infection. In addition to the major whey and casein proteins, also detected in all biological replicates across all experiments were the proteins lactoferrin, lactophorin (glycam-1), osteopontin, polymeric immunoglobulin receptor (PIGR), and butyrophilin. An additional 33 proteins were identified by 2 or more peptides including lactoperoxidase, prothrombin, apolipoprotein A-IV, alpha-1-acid glycoprotein, complement C4, xanthine dehydrogenase, and plasminogen, but the proteins were detected in fewer than 4 biological replicates and thus temporal expression was not evaluated (Supplemental Table 1).

Unlike the previous analysis (Boehmer et al., 2010), the number of spectral counts was greater for most proteins than the number of unique peptides identified, which indicated that the fast scanning speed of the linear ion trap allowed for more accurate relative quantification of a greater number of proteins (Supplemental Table 2). Additionally, though the faster scanning speed of the instrument resulted in lower resolution data, a greater number of proteins were identified, even after low quality spectra were eliminated from the data set (Table 3). As would be expected considering no depletion of high abundance proteins was performed, higher spectral counts

Table 2
Summary of proteins identified in whey from normal or mastitic bovine milk using LC–MS/MS.

Swiss-Prot entry name	Primary accession number	Protein	Condition present[a]	Reference[b]
ALBU_BOVIN	P02769	Serum albumin	N, M	1, 2, 3, 4
LACB_BOVIN	P02754	β-Lactoglobulin	N, M	1, 2, 3, 4
LALBA_BOVIN	P00711	α-Lactalbumin	N, M	1, 2, 3, 4
CASA1_BOVIN	P02662	α-S1-casein	N, M	1, 2, 3, 4
CASA2_BOVIN	P02663	α-S2-casein	N, M	1, 2, 3, 4
CASB_BOVIN	P02666	β-Casein	N, M	1, 2, 3, 4
CASK_BOVIN	P02668	κ-Casein	N, M	1, 2, 3, 4
TRFL_BOVIN	P24627	Lactoferrin	N, M	1, 2, 3, 4
GLCM1_BOVIN	P80195	Glycam-1 (lactophorin)	N, M	1, 2, 4
OSTP_BOVIN	P31096	Osteopontin	N, M	1
PIGR_BOVIN	P81265	Polymeric immunoglobulin receptor	N, M	1, 4
BT1A1_BOVIN	P18892	Butyrophylin	N, M	1
PGRP_BOVIN	Q8SPP7	Peptidoglycan recognition receptor protein	M	1, 2, 3
CTHL1_BOVIN	P22226	Cathelicidin-1	M	1, 2, 3
CTHL2_BOVIN	P19660	Cathelicidin-2	M	1, 3
CTHL4_BOVIN	P33046	Cathelicidin 4	M	1, 3
ACTB_BOVIN	P60712	Actin, cytoplasmic-1	M	1, 4
TRFE_BOVIN	Q29443	Transferrin	M	1, 2, 3, 4
APOA1_BOVIN	P15497	Apolipoprotein A1	M	1, 3
APOA2_BOVIN	P81644	Apolipoprotein A2	M	1, 3
APOE_BOVIN	Q03247	Apolipoprotein E	M	1
APOC3_BOVIN	P109035	Apolipoprotein C3	M	1
APOA4_BOVIN	Q32PJ2	Apolipoprotein A-IV	M	1, 3
APOH_BOVIN	P17690	Beta-2-glycoprotein-1	M	1
VTDB_BOVIN	Q3MHN5	Vitamin D binding protein	M	1
CO3_BOVIN	Q2UVX4	Complement C3	M	1, 3
CFAB_BOVIN	P81187	Complement factor B	M	1
CO4_BOVIN	P01030	Complement C4	M	1, 3
Q693V9_BOVIN	Q693V9	Complement component 3d	M	3
FETUA_BOVIN	P12763	Alpha-2-HS-glycoprotein	M	1, 3
KNG1_BOVIN	P01044	Kininogen 1	M	1, 3
KNG2_BOVIN	P01045	Kininogen 2	M	1
B2MG_BOVIN	P01888	Beta-2-microglobulin	M	1
CLUS_BOVIN	P17697	Clusterin	M	1, 3
SAA_BOVIN	P35541	Serum amyloid A	M	1, 3
ITIH4_BOVIN	Q3T052	Inter-alpha trypsin inhibitor heavy chain 4	M	1
A1AT_BOVIN	P34955	α-1-Antitrypsin	M	3
A1AG_BOVIN	Q3SZR3	Alpha-1-acid glycoprotein	M	1
HPT_BOVIN	Q2TBU0	Haptoglobin	M	1, 3
ITIH1_BOVIN	Q0VCM5	Inter-alpha-trypsin inhibitor heavy chain H1	M	1
FIBA_BOVIN	P02672	Fibrinogen alpha chain	M	1, 3
FIBB_BOVIN	P02676	Fibrinogen beta chain	M	1, 3
FIBG_BOVIN	P12799	Fibrinogen gamma chain	M	1, 3
PERL_BOVIN	P80025	Lactoperoxidase	M	1
PROF1_BOVIN	P02584	Profilin-1	M	1
THRB_BOVIN	P00735	Prothrombin	M	1
A2MG_BOVIN	Q7SIH1	Alpha-2-macroglobulin	M	1, 3
HEMO_BOVIN	Q3SZV7	Hemopexin	M	1
NUCB1_BOVIN	Q0P569	Nucleobindin-1	M	1
ANXA1_BOVIN	P46193	Annexin A1	M	3
CH3L1_BOVIN	P30922	Chitinase-3-like protein 1	M	1
LIPL_BOVIN	P11151	Lipoprotein lipase	M	1
XDH_BOVIN	P80457	Xanthine dehydrogenase/oxidase	M	1
PTGDS_BOVIN	O02853	Prostaglandin-H2 D-isomerase	M	1
PLMN_BOVIN	P06868	Plasminogen	M	1, 3
GELS_BOVIN	Q3SX14	Gelsolin	M	1
FETA_BOVIN	Q3SZ57	Alpha-fetoprotein	M	1
MFGM_BOVIN	Q95114	Lactadherin	M	1
S10A8_BOVIN	P28782	Protein S100-A8	M	1
CAP1_BOVIN	Q3SYV4	Adenyl cyclase-associated protein 1	M	1
ANT3_BOVIN	P41361	Antithrombin-III	M	1, 3
CRF_BOVIN	Q3MHN5	Corticoliberin	M	1
ENOA_BOVIN	Q9XSJ4	Alpha-enolase	M	1
S10A9_BOVIN	P28783	Protein S100-A9 (Calgranulin B)	M	3
Q5J801_BOVIN	Q5J801	Endopin 2B	M	3
PGK1_BOVIN	Q3T0P6	Phosphoglycerate kinase-1	M	1
COF1_BOVIN	Q5E9F7	Cofilin-1	M	1
G3P_BOVIN	P10096	Glyceraldehyde-3-phosphate dehydrogenase	M	1
B4GT1_BOVIN	P08037	β-1,4 Galactosyltransferase-1	M	1
FOLR1_BOVIN	P02702	Folate receptor alpha	M	1
FIT2 BOVIN	A4IFN5	Fat storage-inducing transmembrane protein 2	M	1

[a] Condition present; N = normal bovine milk; M = mastitic bovine milk.
[b] References; 1 = Boehmer et al. (current paper); 2 = Boehmer et al. (2010); 3 = Danielsen et al. (2010); 4 = Smolenski et al. (2007).

Table 3
Summary of proteins detected following the proteomic analysis of normal and mastitic milk.

Swiss-Prot entry name	Primary accession number	Protein[a]	Biological function
Proteins present before and after infection			
ALBU_BOVIN	P02769	Serum albumin	Main plasma protein; binds Ca2+, Na+, K+, fatty acids
LACB_BOVIN	P02754	β-Lactoglobulin	Major whey protein; binds and transports retinol
LALBA_BOVIN	P00711	α-Lactalbumin	Involved in lactose synthesis; regulatory subunit of lactose synthase enzyme
CASA1_BOVIN	P02662	α-S1-casein	Major milk protein; transports calcium phosphate
CASA2_BOVIN	P02663	α-S2-casein	Transports calcium phosphate; inhibits the growth of *E. coli*
CASB_BOVIN	P02666	β-Casein	Acts as a macrophage activator and as a bradykinin-potentiating peptide
CASK_BOVIN	P02668	κ-Casein	Stabilizes micelle formation
TRFL_BOVIN	P24627	Lactoferrin	Iron-binding protein; has antimicrobial activity
GLCM1_BOVIN	P80195	Glycam-1 (Lactophorin)	Mediates trafficking of lymphocytes to lymph nodes
OSTP_BOVIN	P31096	Osteopontin	Enhances production of interferon-gamma and interleukin 12
PIGR_BOVIN	P81265	Polymeric immunoglobulin receptor	Receptor that binds polymeric IgA and IgM at the basolateral surface of epithelial cells
BT1A1_BOVIN	P18892	Butyrophylin	Functions in the secretion of milk-fat droplets
Proteins present only after infection			
PGRP_BOVIN	Q8SPP7	Peptidoglycan recognition receptor protein	Involved in innate immunity; microbicidal for gram (+) and gram (−) bacteria
CTHL1_BOVIN	P22226	Cathelicidin-1	Potent microbicidal activity against *E. coli*
CTHL2_BOVIN	P19660	Cathelicidin-2	Potent antimicrobial activity against *E. coli*
CTHL4_BOVIN	P33046	Cathelicidin 4	Potent microbicidal activity against *E. coli*
ACTB_BOVIN	P60712	Actin, Cytoplasmic-1	Involved in various types of cell motility
TRFE_BOVIN	Q29443	Transferrin	Iron-binding protein
APOA1_BOVIN	P15497	Apolipoprotein A1	Transport protein; major plasma HDL protein
APOA2_BOVIN	P81644	Apolipoprotein A2	Involved in lipid transport; stablizes HDL; has antimicrobial activity
VTDB_BOVIN	Q3MHN5	Vitamin D binding protein	Transport protein; also associates w/immunoglobulins on lymphocytes
CO3_BOVIN	Q2UVX4	Complement C3	Major protein in complement activation
CFAB_BOVIN	P81187	Complement factor B	Part of the alternative complement pathway
FETUA_BOVIN	P12763	Alpha-2-HS-glycoprotein	Promotes endocytosis; has lymphocyte stimulating properties
KNG2_BOVIN	P01045	Kininogen 2	Produces active peptide bradykinin; inflammatory mediator
B2MG_BOVIN	P01888	Beta-2-microglobulin	Beta chain of major histocompatibility complex class I molecules
CLUS_BOVIN	P17697	Clusterin	Function unclear: may be involved in programmed cell death
SAA_BOVIN	P35541	Serum amyloid A	Major acute phase protein
ITIH4_BOVIN	Q3T052	Inter-alpha trypsin inhibitor heavy chain 4	Involved in acute phase reactions
HPT_BOVIN	Q2TBU0	Haptoglobin	Combines w/free plasma hemoglobin; acute phase protein
FIBA_BOVIN	P02672	Fibrinogen alpha chain	Yields monomers that polymerize into fibrin; acts in platelet aggregation
FIBB_BOVIN	P02676	Fibrinogen beta chain	Yields monomers that polymerize into fibrin; acts in platelet aggregation
FIBG_BOVIN	P12799	Fibrinogen gamma chain	Yields monomers that polymerize into fibrin; acts in platelet aggregation

[a] Proteins all identified in 4 or more biological replicates with 2 or more peptide assignments.

per unique peptide identified were detected for the more abundant proteins, while the number of unique peptides identified and the corresponding spectral counts tended to be more equivalent for the lower abundance proteins (Supplemental Table 2). Because spectral counts have been shown to have higher technical reproducibility than peptide counts (Zhang et al., 2006; Old et al., 2005), the temporal expression patterns of proteins identified using nano-LC followed by nano-spray tandem MS were evaluated using spectral counts.

3.3. Relative quantification of proteins related to host response identified in bovine milk

The use of label-free quantification strategies such as number of unique peptide assignments, ion intensity, and spectral counts for the quantification of relative protein abundance has been reported previously (Mosley et al., 2009; McFarland et al., 2008; Florens et al., 2006; Zybailov et al., 2005; Liu et al., 2004). Most previous endeavors have, however, utilized normalized spectral counts to estimate changes in relative abundance of individual proteins present in complex matrices (Mosley et al., 2009; McFarland et al., 2008; Florens et al., 2006; Zybailov et al., 2005). In our previous analysis in which we utilized number of unique peptide assignments to track changes in the expression of proteins detected in whey from milk over the course of a clinical mastitis infection (Boehmer et al., 2010), normalization had little effect on the data due to the minimal sensitivity of the analyses. For the data generated using a linear ion trap, however, raw spectral counts were used to track the temporal expression of proteins present in whey from bovine milk. Normalization was based on digesting the same amount of protein for each sample, and injecting the same volume of digested protein onto the chromatography column for analysis using mass spectrometry. The rationale for normalizing by the amount of protein analyzed versus resulting spectral counts was based on the observation that milk samples collected prior to infection were less complex than later time points, primarily due to the fact that proteins related to host response were absent in the milk from healthy cows. The difference in complexity between 0 h samples and time points following infection was evidenced by fewer proteins identified,

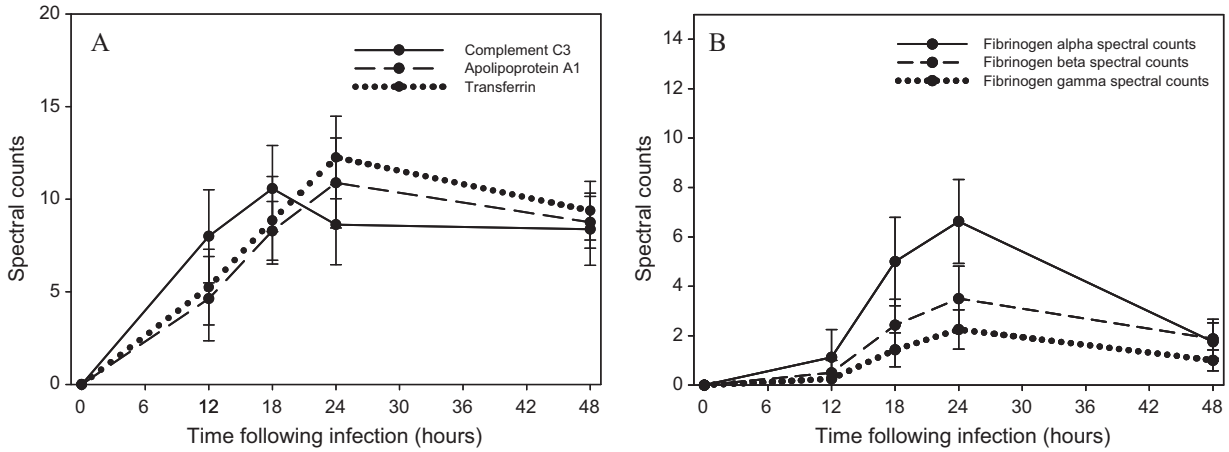

Fig. 3. Temporal expression patterns of vascular derived proteins (mean spectral counts ± standard error) complement C3, apolipoprotein A1, and transferrin (A) and the three different chains of the blood coagulation protein fibrinogen (B). Temporal expression of all the vascular-derived proteins was in accord with previous reports of cytokine expression, in particular IL-1β, and TNFα, which are known to induce vascular leak.

and a much lower number of total spectral counts overall in milk collected from cows prior to inoculation with *E. coli*. Thus, normalization based on the sum of spectral counts at each time point would have resulted in an apparent washout effect of increases in spectral counts between baseline samples and later time points during clinical mastitis.

As was evident in earlier LC–MS/MS analyses (Boehmer et al., 2010; Smolenski et al., 2007), proteins identified in whey from bovine milk at time points following infection using nano-LC–MS/MS were predominantly vascular derived, acute phase, antimicrobial, complement, or related to immune response, and fell into categories that could be broadly classified as secondary effects of cytokine induction. The most abundant vascular-derived proteins identified included complement factor C3, transferrin, and apolipoprotein A3 (Fig. 3A). Somewhat less abundant were the fibrinogen proteins that are known to be involved in blood coagulation (Fig. 3B). The vascular-derived proteins identified were all clearly biologically relevant, as firmly established hallmarks of coliform mastitis include the induction of cytokines IL-1β and TNFα, which cause fever, complement activation, hepatic production of acute phase proteins (Suojala et al., 2008; Grönlund et al., 2005; Dinarello, 1996), and the leakage of plasma proteins and complement factors into the milk (Bannerman et al., 2004, 2008; Riollet et al., 2000; Shuster et al., 1997). Biological relevance was further evidenced by the fact that the majority of proteins present only in whey from milk collected following infection exhibited the greatest increases in total spectral counts between 18 h and 24 h after inoculation (Supplemental Table 2), which corresponds to peak cytokine expression previously reported to occur between 16 and 24 h during coliform mastitis (reviewed in Bannerman, 2009).

Temporal expression determined using spectral counts also corresponded well with increases in milk somatic cell

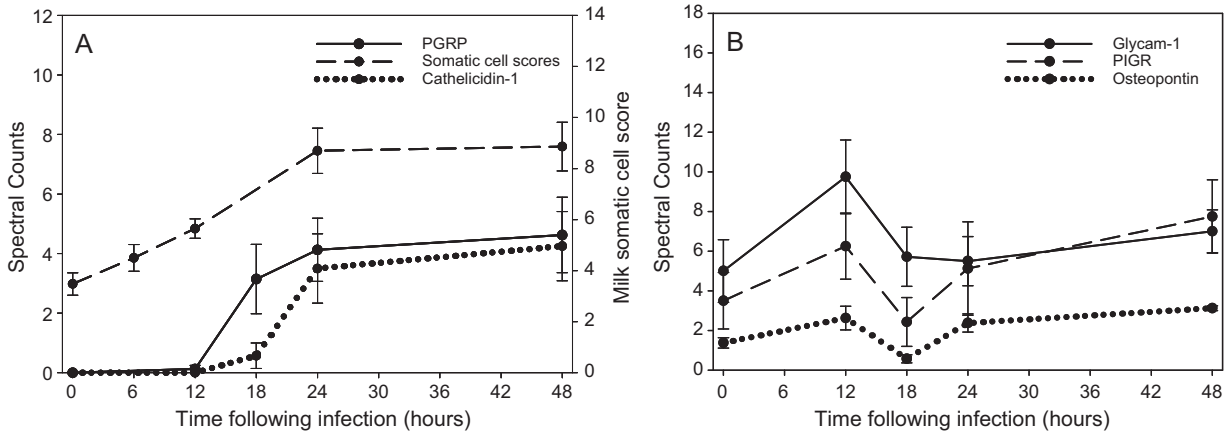

Fig. 4. Temporal expression patterns of antimicrobial proteins (mean spectral counts ± standard error) detected only in time points following inoculation with *E. coli* (A) and glycam-1, PIGR, and osteopontin, proteins present in milk before (Time 0) and after infection (B). Antimicrobial proteins exhibited similar expression patterns to milk somatic cell counts (MSCC), which are comprised primary of neutrophils during infections, and are an established source of the proteins with antimicrobial properties. The correspondence of increases in MSCC to increases in the abundance of antimicrobial proteins indicates that LC–MS/MS label-free data can accurately model clinical indications of coliform mastitis.

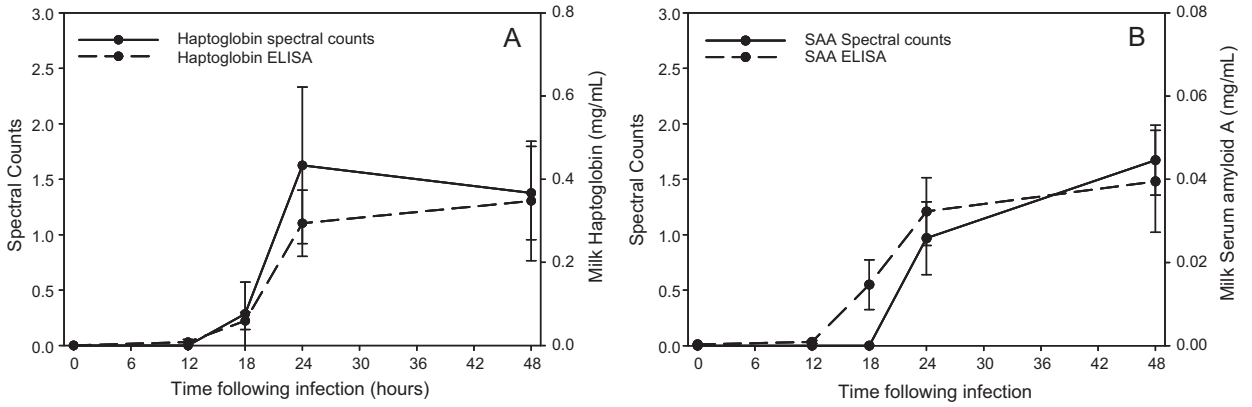

Fig. 5. Comparison of temporal expression patterns of low abundance acute phase proteins determined using ELISA and total spectral counts (mean spectral counts ± standard error) for (A) milk haptoglobin and (B) milk serum amyloid A. Though sensitivity levels differ, the correspondence of the overall patterns exhibited by the LC–MS/MS data and the ELISA data indicates that spectral counts can be used as a screening tool to profile changes in biologically relevant proteins without a reliance on antibodies.

counts (MSCC; Fig. 4A), which are comprised primarily of PMN during intramammary infection (Paape et al., 1981), and are also a well established source of antimicrobial proteins including peptidoglycan recognition receptor protein (PGRP), and members of the cathelicidin family of cationic antimicrobial peptides (Tydell et al., 2006; Scocchi et al., 1997). Though the identifications of the antimicrobial peptides (AMP) were not as robust as more abundant proteins (Supplemental Table 2), the current proteomic analysis marks the first evaluation of the temporal expression of AMP during coliform mastitis, and the first indication that spectral counts can accurately model clinical signs of the disease.

In contrast, some proteins identified, including glycam-1, PIGR, and osteopontin, exhibited decreases in total spectral counts at 18 h following infection (Fig. 4B). The increased dynamic range of proteins present in the milk samples at 18 h following infection due to the influx of vascular-derived proteins and other proteins related to host response is one possible explanation for the apparent decrease in the detection of constitutively expressed milk proteins. However, glycam-1, PIGR, and osteopontin are all known to be subject to glycosylation and phosphorylation, and thus heavy modifications at 18 h following infection is a more plausible explanation for the apparent decrease in all 3 proteins as was evidenced by a decline in their respective total spectral counts (Kjeldsen et al., 2003; Soerensen et al., 1995).

3.4. LC–MS/MS label free quantification correlates with ELISA data

To the authors' knowledge, no other proteomic evaluation of bovine milk has assessed the temporal expression of proteins identified in milk over time, nor compared LC–MS/MS data with ELISA data to assess potential accuracy. The accuracy of LC–MS/MS label-free strategies in tracking changes in the relative abundance of proteins in milk during clinical mastitis was, however, evaluated in our earlier proteomic analysis of milk by comparison of ELISA data to LC–MS/MS peptide count data for abundant milk proteins (Boehmer et al., 2010). After changing instrument systems, however, a greater number of low abundance proteins related to host response were detected in whey from mastitic milk (Table 3), allowing us to evaluate the accuracy of LC–MS/MS label-free quantification of minor proteins when compared with antibody-based strategies. Comparisons of ELISA data and total spectral counts for milk Hp (Fig. 5A) and SAA (Fig. 5B) revealed trends in temporal expression with very similar overall patterns. Biological variability was apparent, but the peptides identified for each protein were uniform among samples, and trends in total spectral counts tracked both across the biological replicates and with ELISA data (Supplemental Table 2). The comparison of ELISA data from low abundance acute phase proteins with total spectral counts indicated slight advantages in sensitivity with ELISA compared to spectral counts, in that the antibody-based analysis was able to detect the presence of acute phase proteins in milk at earlier time points than mass spectrometry. However, the fact that peptides from relatively low abundance acute phase proteins were detected in an extremely biologically complex matrix using LC–MS/MS despite the lack of sample fractionation and only a one-dimensional LC separation, further establishes the feasibility of using spectral counts to track changes in proteins related to host response, especially those for which no antibody or ELISA currently exists.

3.5. Temporal expression of proteins not previously identified in bovine milk

Previous proteomic analyses of whey from mastitic bovine milk (Danielsen et al., 2010; Boehmer et al., 2008, 2010; Smolenski et al., 2007; Hogarth et al., 2004) have identified a number of low abundance proteins related to the host response in bovine milk (Table 2). The comparative analysis of whey from bovine milk following experimental induction of coliform mastitis accomplished using one dimensional nano-LC and a linear ion trap mass spectrometer, however, identified a small number of potentially novel biomarkers of coliform mastitis not previously reported including the acute phase protein ITIH4, the eicasonoid

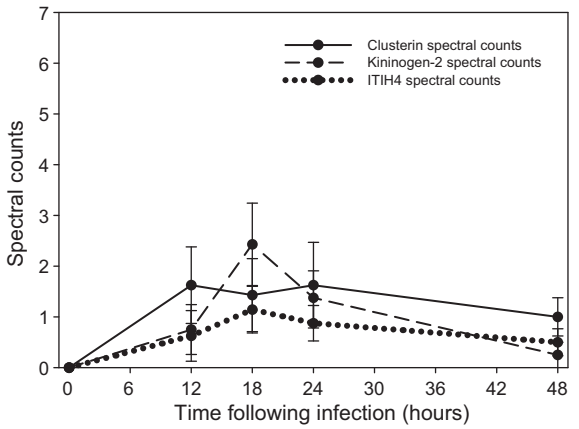

Fig. 6. The temporal expression of the previously uncharacterized proteins clusterin, kininogen-2, and ITIH4 (mean spectral counts ± standard error) detected in whey from mastitic milk. The roles of clusterin, kininogen-2, and ITIH4 during coliform mastitis are currently not known, but the results represent the advantage of using LC–MS/MS label-free strategies to screen for biologically relevant candidate biomarkers, without the use of antibodies or costly labeling reagents.

precursor kininogen-2, several apolipoproteins, and the poorly characterized protein clusterin (Table 3). Similar to the vascular-derived proteins, ITIH4, kininogen-2, and clusterin all appeared to be biologically relevant, as their patterns of modulation (Fig. 6) corresponded with previous reports of peak cytokine expression during coliform mastitis (reviewed in Bannerman, 2009). Prior reports of ITIH4 in cattle have been limited to isolation of the APP from the serum of heifers with experimentally induced summer mastitis (Pineiro et al., 2004). Furthermore, ITIH4 has never been identified in bovine milk, and has never been associated with coliform mastitis in lactating dairy cattle. The association of ITIH4 with innate immunity has, however, been studied in models of acute inflammation in swine (Gonzalez-Ramon et al., 2000), and ITIH4 was recently reported to be a novel marker of acute ischemic stroke in humans (Kashyap et al., 2009). Previous research and similarity to a human homolog led to the classification of ITIH4 as a plasma kallikrein-sensitive glycoprotein, but the exact role and function of ITIH4 in the bovine mammary gland during inflammation associated with coliform mastitis is not yet clear (Nishimura, 2003).

Kininogen-2, on the other hand, belongs to the family of plasma kallikreins, or serine proteases, known to play key roles in blood coagulation (Furie and Furie, 1988), fibrinolysis (Ichinose et al., 1984), activation of complement (Discipio, 1982), and the release of bradykinin from its precursor, kininogen (Scharfstein et al., 2007). The kinin peptides, including bradykinin, are potent mediators of vasodilation, pain, and udder edema during clinical mastitis (Eshraghi et al., 1999). The relationship between elevated levels of milk bradykinin and increased severity of clinical symptoms of mastitis has been demonstrated, but bradykinin levels in milk from cows with experimentally induced coliform mastitis have not yet been investigated (Eshraghi et al., 1999). Likewise, while the activation of complement and the cleavage of complement proteins into active peptides that further enhance the inflammatory response have been documented during coliform mastitis (Suojala et al., 2008; Grönlund et al., 2005; Shuster et al., 1997), less is known about the contributions of the kallikrein–kinin system to inflammation in the bovine mammary gland.

Apolipoproteins are typically associated with high density lipoproteins (HDLs), but other roles for the apolipoproteins during disease, and inflammation in particular, have been investigated (Burger and Dayer, 2002; Cockerill et al., 2001; Bausserman et al., 1988). Though some apolipoproteins have been identified in previous proteomic analyses of bovine milk (Danielsen et al., 2010; Boehmer et al., 2008, 2010; Smolenski et al., 2007), the role of the apolipoproteins during inflammation, and coliform mastitis specifically, has not yet been determined. Similarly, the function of clusterin during coliform mastitis is entirely unclear; however, the involvement of clusterin in several inflammatory diseases including myocarditis, and glomerulopathy has been implicated from previous research (Rosenberg et al., 2002; McLaughlin et al., 2000). Results of prior research likewise indicate that clusterin could possess anti-inflammatory properties (Rosenberg et al., 2002; McLaughlin et al., 2000). In terms of the role of clusterin in the bovine mammary gland, all that has been inferred is that clusterin could be associated with mammary gland involution, the clearance of cellular debris, and apoptotic cell death (Jones and Jomary, 2002).

3.6. Temporal expression of proteins yields quantitative and qualitative data

Variable response to experimental infection will always be a factor when using a bovine *in vivo* challenge model for biomarker discovery analyses. In some cases, however, while biological variation may prevent accurate quantification, the apparent deviation of proteins identified in milk from one animal, compared to the rest of the biological replicates, could provide useful qualitative data. In the comparative proteomic analysis of whey from bovine milk that utilized one-dimensional nano-LC followed by nano-spray tandem MS, although biological variability was apparent across all cows, the biological replicates with patterns of total spectral counts that most often did not track with the rest of the subjects were cows 3 and 6 (Supplemental Table 2). The most interesting aspect regarding proteins identified in milk from cows 3 and 6 is the nearly complete absence of proteins related to host response, other than vascular-derived proteins, in samples collected from both cows following infection. Markedly higher total spectral counts for serum albumin and lactoferrin in the baseline milk sample collected from cow 3, when compared to other biological replicates, could indicate that cow 3 had recently resolved a naturally occurring clinical infection, and that despite otherwise normal clinical parameters, milk levels of serum albumin and lactoferrin had not yet returned to baseline at the time of the challenge. Cow 6, on the other hand, had baseline spectral count values for serum albumin and transferrin that were in accord with the other biological replicates, but total spectral counts for vascular-derived proteins such as complement C3, apolipoprotein A1, transferrin, and the fib-

rinogens that did not increase above baseline values until 48 h following infection. The indication, based on proteins detected and total spectral count patterns, is that cow 6 was perhaps much slower to respond to challenge than the other cows included on the study. While analytical errors could explain the somewhat aberrant results for cows 3 and 6, the results could also indicate that with further refinements, including the use of both biological and technical replicates and 2D-LC separations, biologically relevant information of a more qualitative nature, including variation in response to challenge or infection status, could be obtained from proteomic milk protein expression patterns.

4. Conclusion

Clearly, several challenges still remain regarding the identification and accurate quantification of biomarkers of host response in bovine milk following experimental induction of coliform mastitis. While results of comparative proteomic analyses have revealed promising candidate biomarkers, biological complexity of bovine milk, both before and following challenge, as well as the inherent variability apparent across biological replicates, has precluded the establishment of a biomarker or pattern of biomarkers specific to coliform mastitis. Nonetheless, the results of the proteomic analysis conducted to date have provided information that could prove useful in the design and execution of future studies of inflammatory biomarkers in bovine milk, including potential candidates for more focused follow-up analyses, and more in-depth knowledge of the suitability of different methodologies and the capability of different instrument systems. A significant contribution of more recent analyses that included the evaluation of a longitudinal set of milk samples collected from 8 cows over the course of clinical infection, is the more biologically robust identification of several relevant proteins related to host response in the bovine mammary gland detected following the use of nano-LC, nano-spray ionization, and a linear ion trap mass spectrometer. The proteins that stand out as logical candidates for follow-up analyses include the various APP and AMP detected, vascular-derived proteins such as the apolipoproteins, and proteins previously unaffiliated with coliform mastitis including clusterin, ITIH4 and kininogen-2, a bradykinin precursor. Additionally, the evaluation of spectral counts as a means of tracking temporal expression of milk proteins during coliform mastitis demonstrated that despite inherent flaws, biological variability, and the current lack of supporting statistical analysis, spectral counts appear to be an effective means of illustrating protein modulation during clinical infections. Given the fact that a majority of previous reports dealing with the expression of inflammatory mediators during coliform mastitis have used data derived from ELISAs, and that there is currently only a limited number of commercially available bovine-specific antibodies, proteomic strategies designed to discover or characterize novel or poorly understood proteins related to host response afford clear advantages over traditional antibody-based methods.

5. Conflict of interest statement

The authors of this manuscript have no financial, contractual, or personal relationships with any other persons, organizations, or entities that could influence or bias the nature of the research presented.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2010.10.004.

References

Angal, S., Dean, P.D.G., 1977. The effect of matrix on the binding of albumin to immobilized cibacron blue. Biochem. J. 167, 301–303.

Bannerman, D.D., 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87 (13 Suppl.), 10–25.

Bannerman, D.D., Kauf, A.C., Paape, M.J., Springer, H.R., Goff, J.P., 2008. Comparison of Holstein and Jersey innate immune responses to *Escherichia coli* intramammary infection. J. Dairy Sci. 91 (6), 2225–2235.

Bannerman, D.D., Paape, M.J., Lee, J.-W., Zhao, X., Hope, J.C., Rainard, P., 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab Immunol. 11 (3), 463–472.

Bausserman, L.L., Bernier, D.N., McAdam, K.P.W.J., Herbert, P.N., 1988. Serum amyloid A and high density lipoproteins during the acute phase response. Eur. J. Clin. Invest. 18, 619–626.

Boehmer, J.L., Ward, J.L., Peters, R.R., Shefcheck, K.J., McFarland, M.A., Bannerman, D.D., 2010. Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification. J. Dairy Sci. 93 (2), 593–603.

Boehmer, J.L., Bannerman, D.D., Shefcheck, K.J., Ward, J.L., 2008. Proteomic analysis of differentially expressed proteins in bovine milk during experimentally induced *Escherichia coli* mastitis. J. Dairy Sci. 91 (11), 4206–4218.

Burger, D., Dayer, J.-M., 2002. High-density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? Autoimmun. Rev. 1, 111–117.

Cockerill, G.W., Huehns, T.Y., Weerasinghe, A., Stocker, C., Lerch, P.G., Miller, N.E., Haskard, D.O., 2001. Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an in vivo model of acute inflammation. Circulation 103, 108–112.

Colantonio, D.A., Chan, D.W., 2005. The clinical application of proteomics. Clin. Chim. Acta 357, 151–158.

Danielsen, M., Codrea, M.C., Ingvartsen, K.L., Friggens, N.C., Bendixen, E., Røntved, C.M., 2010. Quantitative milk proteomics – Host responses to lipopolysaccharide-mediated inflammation of bovine mammary gland. Proteomics (Epub ahead of print).

Dinarello, C.A., 1996. Biological basis for interleukin-1 in disease. Blood 87 (6), 2095–2147.

Discipio, R.G., 1982. The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology 45 (3), 587–595.

Eckersall, P.D., Young, F.J., McComb, C., Hogarth, C.J., Safi, S., Weber, A., McDonald, T., Nolan, A.M., Fitzpatrick, J.L., 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet. Rec. 148, 35–41.

Eshraghi, H.R., Zeitlin, I.J., Fitzpatrick, J.L., Ternent, H., Logue, D., 1999. The release of bradykinin in bovine mastitis. Life Sci. 64 (18), 1675–1687.

Fenselau, C., 2007. A review of quantitative methods for proteomic studies. J. Chromatogr. B. 855, 14–20.

Florens, L., Carozza, M.J., Swanson, S.K., Fournier, M., Coleman, M.K., Workman, J.L., Washburn, M.P., 2006. Analyzing chromatin remodeling complexes using shotgun proteomics and normalized spectral abundance factors. Methods 40 (4), 303–311.

Furie, B., Furie, B.C., 1988. The molecular basis of blood coagulation. Cell 53 (4), 505–518.

Gagnaire, V., Jardin, J., Jan, G., Lortal, S., 2009. Invited review: proteomics of milk and bacteria used in fermented dairy products: from qualitative to quantitative advances. J. Dairy Sci. 92 (3), 811–825.

Gonzalez-Ramon, N., Hoebe, K., Alava, M.A., van Leengoed, L., Pineiro, M., Carmona, S., Iturralde, M., Lampreave, F., Pineiro, A., 2000. Pig MAP/ITIH4 and haptoglobin are interleukin-6-dependent acute-phase plasma proteins in porcine primary cultured hepatocytes. Eur. J. Biochem. 267 (6), 1878–1885.

Grönlund, U., Hallén Sandgren, C., Persson Waller, K., 2005. Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. Vet. Res. 36 (2), 191–198.

Gu, S., Pan, S., Bradbury, E.M., Chen, X., 2002. Use of deuterium-labeled lysine for efficient protein identification and peptide de novo sequencing. Anal. Chem. 74 (22), 5774–5785.

Gundry, R.L., Fu, Q., Jelinek, C.A., Van Eyk, J.E., Cotter, R.J., 2007. Investigation of an albumin-enriched fraction of human serum and its albuminome. Proteomics Clin. Appl. 1, 73–88.

Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., Aebersold, R., 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–999.

Hiss, S., Mielenz, M., Bruckmaier, M., Sauerwein, H., 2004. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J. Dairy Sci. 87, 3778–3784.

Hogarth, C.J., Fitzpatrick, J.L., Nolan, A.M., Young, F.J., Pitt, A., Eckersall, P.D., 2004. Differential protein composition of bovine whey: a comparison of whey from healthy animals and from those with clinical mastitis. Proteomics 4 (7), 2094–2100.

Ichinose, A., Kisiel, W., Fujikawa, K., 1984. Proteolytic activation of tissue plasminogen activator by plasma and tissue enzymes. FEBS Lett. 175 (2), 412–418.

Jiang, H., English, A.M., 2002. Quantitative analysis of the yeast proteome by incorporation of isotopically labeled leucine. J. Proteome Res. 1 (4), 345–350.

Jones, S.E., Jomary, C., 2002. Clusterin. Int. J. Biochem. Cell Bio. 34, 427–431.

Juraschek, R., Dülks, T., karas, M., 1998. Nanoelectrospray – more than just a minimized-flow electrospray ionization source. J. Am. Soc. Mass Spectrom. 10, 300–308.

Kashyap, R.S., Nayak, A.R., Deshpande, P.S., Kabra, D., Purohit, H.J., Taori, G.M., Daginawala, H.F., 2009. Inter-α-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. Clin. Chim. Acta 402, 160–163.

Kjeldsen, F., Haselmann, K.F., Budnik, B.A., Sorensen, E.S., Zubarev, R.A., 2003. Complete characterization of posttranslational modification sites in the bovine milk protein PP3 by tandem mass spectrometry with electron capture dissociation as the last stage. Anal. Chem. 75, 2355–2361.

Lippolis, J.D., Bayles, D.D., Reinhardt, T.A., 2009. Proteomic changes in *Escherichia coli* when grown in fresk milk versus laboratory media. J. Proteome Res. 8 (1), 149–158.

Liu, H., Sadygov, R.G., Yates III, J.R., 2004. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. 76 (14), 4193–4201.

Mann, M., Hendrickson, R.C., Pandey, A., 2001. Analysis of proteins and proteomes by mass spectrometry. Annu. Rev. Biochem. 70, 437–473.

McFarland, M.A., Ellis, C.E., Markey, S.P., Nussbaum, R.L., 2008. Proteomics analysis identifies phosphorylation-dependent α-synuclein protein interactions. Mol. Cell. Prot. 7 (11), 2123–2137.

McLaughlin, L., Zhu, G., Mistry, M., Ley-Ebert, C., Stuart, W.D., Florio, C.J., Groen, P.A., Witt, S.A., Kimball, T.R., Witte, D.P., Harmony, J.A.K., Aronow, B.J., 2000. Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. J. Clin. Invest. 106, 1105–1113.

Mosley, A.L., Florens, L., Wen, Z., Washburn, M.P., 2009. A label free quantitative proteomic analysis of the *Saccharomyces cerevisiae* nucleus. J. Proteomics 72 (1), 110–120.

Mueller, L.N., Brusniak, M.-Y., Mani, D.R., Aebersold, R., 2008. An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. J. Proteome Res. 7 (1), 51–61.

Nishimura, T., 2003. Expression of potential lymphocytes trafficking mediator molecules in the mammary gland. Vet. Res. 34, 3–10.

Oda, Y., Huang, K., Cross, F.R., Cowburn, D., Chait, B.T., 1999. Accurate quantitation of protein expression and site-specific phosphorylation. Proc. Natl. Acad. Sci. U.S.A. 96 (12), 6591–6596.

O'Donnell, R., Holland, J.W., Deeth, H.C., Alewood, P., 2004. Review: milk proteomics. Int. Dairy J. 14, 1013–1023.

Old, W.M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K.G., Mendoza, A., Sevinsky, J.R., Resing, K.A., Ahn, N.G., 2005. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol. Cell. Prot. 4 (10), 1487–1502.

Paape, M.J., Wergin, W.P., Guidry, A.J., Schultze, W.D., 1981. Phagocytic defense of the ruminant mammary gland. Adv. Exp. Med. Biol. 137, 555–578.

Pineiro, M., Andres, M., Iturralde, M., Carmona, S., Hirvonen, J., Pyorala, S., Heegard, P.M.H., Tjornehoj, K., Lampreave, F., Pineiro, A., Alava, M.A., 2004. ITIH4 (Inter-alpha-trypsin inhibitor heavy chain 4) is a new acute-phase potein isolated from cattle during experimental infection. Infect. Immun. 72 (7), 3777–3782.

Reinhardt, T.A., Lippolis, J.D., 2008. Developmental changes in the milk fat globule membrane proteome during the transition from colostrums to milk. J. Dairy Sci. 91, 2307–2318.

Riollet, C., Rainard, P., Poutrel, B., 2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. Clin. Diagn. Lab. Immunol. (2), 161–167.

Roe, M.R., Griffin, T.J., 2006. Gel-free mass spectrometry-based high throughput proteomics: tools for studying biological response of proteins and proteomes. Proteomics 6, 4678–4687.

Rosenberg, M.E., Girton, R., Finkel, D., Chmielewski, D., Barrie III, A., Witte, D.P., Zhu, G., Bissler, J.J., Harmony 2, J.A.K., Aronow, B.J., 2002. Apolipoprotein J/clusterin prevents a progressive glomerulopathy of aging. Mol. Cell. Bio. 22, 1893–1902.

Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., Pappin, D.J., 2004. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Prot. 3, 1154–1169.

Scharfstein, J., Schmitz, V., Svensjo, E., Granato, A., Monteiro, A.C., 2007. Kininogens coordinate adaptive immunity through the proteolytic release of bradykinin, an endogenous danger signal driving dendritic cell maturation. Scand. J. Immunol. 66 (2–3), 128–136.

Scocchi, M., Wang, S., Zanettia, M., 1997. Structural organization of the bovine cathelicidin gene family and identification of a novel member. FEBS Lett. 417 (3), 311–315.

Shuster, D.E., Kehrli, M.E., Rainard, P., Paape, M., 1997. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. Infect. Immun. 65 (8), 3286–3292.

Simpson, K., Whetton, A.D., Dive, C., 2009. Quantitative mass spectrometry-based techniques for clinical use: biomarker identification and quantification. J. Chromatogr. B. 877 (13), 1240–1249.

Smolenski, G., Haines, S., Kwan, Y.-S., Bond, F., Farr, J., Davis, V.S.R., Stelwagen, K., Wheeler, T.T., 2007. Characterization of host defense proteins in milk using a proteomic approach. J. Proteome Res. 6 (1), 207–215.

Soerensen, E.S., Hoejrup, P., Petersen, T.E., 1995. Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites. Protein Sci. 4, 2040–2049.

Suojala, L., Orro, T., Järvinen, H., Saatsi, J., Pyörälä, S., 2008. Acute phase response in two consecutive experimentally induced E. coli intramammary infection in dairy cows. Acta Vet. Scand. 50 (June (13)), 18 (Epub.).

Tydell, C.C., Yuan, J., Tran, P., Selsted, M.E., 2006. Bovine peptidoglycan recognition protein-S: antimicrobial activity, localization, secretion, and binding properties. J. Immunol. 176 (2), 1154–1162.

Wilm, M., Mann, M., 1996. Analytical properties of the nanoelectrospray ion source. Anal. Chem. 68 (1), 1–8.

Yamada, M., Murakami, K., Wallingford, J., Yuki, Y., 2002. Identification of low-abundance proteins of bovine colostral and mature milk using two-dimensional electrophoresis followed by microsequencing and mass spectrometry. Electrophoresis 23 (7–8), 1153–1160.

Yao, X., Afonso, C., Fenselau, C., 2003. Dissection of proteolytic 18O labeling: endoprotease-catalyzed 16O-to-18O exchange of truncated peptide substrates. J. Proteome Res. 2, 147–152.

Zhang, B., VerBerkmoes, N.C., Langston, M.A., Uberbacher, E., Hettich, R.L., Samatova, N.F., 2006. Detecting differential and correlated protein expression in label-free shotgun proteomics. J. Proteome Res. 5, 2909–2918.

Zybailov, B., Coleman, M.K., Florens, L., Washburn, M.P., 2005. Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal. Chem. 77 (19), 6218–6224.